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Development and Application of an Assay for Uranyl Complexation by Fungal Metabolites, Including Siderophores

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An assay to detect UO_2^{2+} complexation was developed based on the chrome azurol S (CAS) assay for siderophores (B. Schwyn and J. B. Neilands, Anal. Biochem. 160:47-56, 1987) and was used to investigate the ability of fungal metabolites to complex actinides. In this assay the discoloration of two dyed agars (one containing a CAS-Fe^{3+} dye and the other containing a CAS-UO_2^{2+} dye) caused by ligands was quantified. The assay was tested by using the siderophore desferrioxamine B (DFO), and the results showed that there was a regular, reproducible relationship between discoloration and the amount of siderophore added. The ratio of the discoloration on the CAS-UO_2^{2+} agar to the discoloration on the CAS-Fe^{3+} agar was independent of the amount of siderophore added. A total of 113 fungi and yeasts were isolated from three soil samples taken from the Peak District National Park. The fungi were screened for the production of UO_2^{2+} chelators by using the CAS-based assay and were also tested specifically for hydroxamate siderophore production by using the hydroxamate siderophore auxotroph *Aureobacterium flavescens* JG-9. This organism is highly sensitive to the presence of hydroxamate siderophores. However, the CAS-based assay was found to be less sensitive than the *A. flavescens* JG-9 assay. No significant difference between the results for each site for the two tests was found. Three isolates were selected for further study and were identified as two *Pencillium* species and a *Mucor* species. Our results show that the new assay can be effectively used to screen fungi for the production of UO_2^{2+} chelating ligands. We suggest that hydroxamate siderophores can be produced by mucoraceous fungi.

Siderophores are relatively low-molecular-mass (500- to 1,000-Da), iron-chelating ligands that are synthesized by most microorganisms under iron-limited conditions (22, 34, 64, 65) and usually contain catecholate or hydroxamate functional groups (8, 60). Siderophores are highly specific for Fe^{3+} (30, 35) but have been found to bind effectively to actinides, such as thorium, uranium, neptunium, and plutonium (9, 40, 47). These elements are of concern since they are released into the environment as a result of nuclear weapons production and testing and nuclear fuel cycle operations (15, 16). Siderophores and other naturally occurring ligands may therefore affect actinide mobility in waste repositories and the environment and may also be used to treat radioactive waste prior to storage or to decontaminate soils and waters (15, 24, 42, 56). Siderophores and siderophore analogues have also been investigated for possible medical use in the decorporation of actinides from the body (25, 48, 49), while simple hydroxamates may have uses in the reprocessing of nuclear fuel (31, 32, 51, 52).

In this study we investigated the ability of fungal hydroxamate siderophores to act as ligands for actinides. Although fungal siderophores are almost exclusively of the hydroxamate type, they have great molecular structural diversity. An assay to detect actinide complexation was developed based on the chrome azurol S (CAS) assay for siderophores of Schwyn and Neilands (44). In the CAS assay a highly colored complex formed by CAS, hexadecyltrimethylammonium cation (HDTMA), and Fe^{3+} is utilized. When Fe^{3+} is removed from the complex by another ligand (e.g., a siderophore), the dye changes color from blue to orange-pink. The assay developed here was used to screen unidentified soil isolates for the production of UO_2^{2+} chelators. Fungi were isolated from soil samples taken from the Peak District National Park in the United Kingdom, and the isolates were grown in iron-limited liquid cultures to induce siderophore production. In addition to this assay for UO_2^{2+} complexation, the culture supernatant was also tested by using the *Aureobacterium flavescens* JG-9 assay (38, 39, 50) for hydroxamate siderophore production. From this screening analysis of the soil isolates, three isolates which produced UO_2^{2+} selective complexants were identified to the genus level.

MATERIALS AND METHODS

Soil organisms. Samples of soil (~100 g) were collected from 10 to 20 cm below the surface at three sites (sites A, B, and C) in Peak District National Park in the United Kingdom and were stored in sealed plastic containers at 4°C. Organisms were isolated from the soil samples within 2 days of sampling on agar-solidified Vogel's medium (55) supplemented with thiamine (2 mg $liter^{-1}$), penicillin (100,000 U $liter^{-1}$), and streptomycin (100 mg $liter^{-1}$), and the pH was adjusted to either pH 7.0 to 7.5 or pH 4.5 to 5, depending on the pH of the original soil (see below). The cultures were incubated at 20°C for 4 to 7 days. A total of 113 isolates were collected and stored on the isolation medium at 4°C. Three organisms (A1.8, B2.14b, and C2.47) that were selected for further study were maintained at 25°C on agar-solidified Vogel's medium supplemented with

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thiamine (2 mg liter^{-1}) or on agar-solidified medium containing (per liter) 3 g of malt extract, 3 g of yeast extract, 5 g of mycological peptone, and 10 g of glucose.

To induce siderophore production, soil isolates were grown in 25-ml universal tubes containing 5 ml of Vogel's medium (pH 4.5 to 5.0 or pH 7.0 to 7.5) with ferrous ammonium sulfate and sodium citrate omitted and the potassium dihydrogenphosphate concentration reduced to 30 mg liter^{-1}. The neutral medium was buffered with 50 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane. The universal tubes were sealed with sterile foam bungs and incubated at 25°C for 5 to 13 days. The culture supernatants were collected and sterilized by using a 0.2-μm-pore-size filter.

Analysis of soil samples. The method used to measure the pH of the soil samples was based on the method of Forster (12). Soil was dried overnight at ~65°C and disaggregated with a pestle and mortar, and 10 ml was added to 100 ml of dialyzed water. The soil suspension was stirred vigorously for 2 min and then left to stand for 1 h. It was then stirred vigorously for a further 2 min, and the pH was measured. To prepare the soil samples for measurement of the iron content, the samples were dried to a constant weight, disaggregated with a pestle and mortar, and then passed through a 63-μm-pore-size sieve. The iron concentrations of the soil samples were determined by M. Jennings (Department of Chemistry, University of Manchester).

***A. flavescens* JG-9 assay.** *A. flavescens* JG-9 (= ATCC 25091) was maintained at 30°C on agar-solidified medium containing (per liter) 10 g of peptone, 10 g of yeast extract, 2 g of K_2HPO_4, and 38 μg of desferrioxamine B mesylate (DFO) and was stored in 20% (vol/vol) aqueous glycerol at −80°C.

The assay medium was the same as the medium used to maintain *A. flavescens* JG-9 but with DFO omitted and the bacterium added at low levels. A bacterial suspension was added to the molten medium until the medium just started to turn cloudy (5 to 10 ml liter^{-1}). Filter-sterilized supernatant samples (50 μl) from the soil isolates in a low-iron culture were added to wells cut in the agar-solidified *A. flavescens* JG-9 assay medium and incubated at 30°C for 4 days.

Assay for comparison of UO_2^{2+} and Fe^{3+} complexation. Two sets of petri dishes were prepared. One set contained CAS-Fe^{3+} · HDTMA dye agar, and the other contained CAS-UO_2^{2+} · HDTMA dye agar. All glassware was washed with ~6 M HCl and rinsed with dialyzed water. HDTMA was dried at 60°C overnight. Dialyzed water was used throughout. HDTMA (72.9 mg) was dissolved in ~300 ml of water. CAS (60.5 mg) was washed into a 500-ml volumetric flask, and 20 ml of an $FeCl_3$ or UO_2Cl_2 solution (1 mM) was added. The HDTMA solution was added slowly to the flask with mixing. The dye solution was then made up to 500 ml and transferred to a 2-liter conical flask. With this method it is essential for both dyes to be present in the agar at the same concentrations in all of the petri dishes prepared. To ensure this, the volumetric flask used to prepare the dye was rinsed with 500 ml of buffer solution (54 mM 4-morpholinoethanesulfonic acid, pH 6.5). The buffer solution and 15 g of agar were then added to the dye solution and autoclaved at 121°C for 15 min. It was important that all of the petri dishes contained the same amount of agar, so a Jencons Scientific sequential peristaltic autodispenser was used to dispense the dye agars (~250 ml) into the square petri dishes (22 by 22 cm). The dishes were left to set on a level surface to ensure that the layer of agar was even throughout, and then wells (nine wells per dish) were cut into the agar.

To test the relationship between the discoloration of the dyed agars and the amount of ligand added, DFO solutions (200 μl; 0.1 to 1 mM) were added to the wells. To test the effect of acid on the dyed agars, DFO solutions (100 μl; 0.1 to 1.0 mM), citric acid solutions (100 μl; 1 to 100 mM), and hydrochloric acid (100 μl; 0.01 to 1.00 M) were added to the wells. To screen the soil isolates, supernatants (200 μl) from the iron-limited universal cultures were added to the wells. The dishes were left at room temperature for ~1 week.

Image analysis. Digital images of the petri dishes containing dye agars were obtained by using a Leaf Systems Inc. Lumina digital camera. An example is shown in Fig. 1a. The images were then analyzed by using the UTHSCSA ImageTool program (available from http://ddsdx.uthscsa.edu/dig/itdesc.html) to quantify the pink discoloration (halo) around each well. First the image was converted to greyscale (Fig. 1b). Two line profiles were then measured across each halo, one horizontally and the other vertically, as indicated in Fig. 1b. The line profile measured the grey level of the image. An example of a line profile is shown in Fig. 1c. The grey level was higher for the discolored pink region than for the blue region. The line profile was saved as a text file listing the x and y coordinates and the grey level for each point along the line profile and then was imported into the Microsoft Excel program. The increase in grey level ($G_{inc.}$) was calculated for each point. $G_{inc.}$ was defined as follows: $G_{inc.} = G_{x,y} - G_{blue}$, where $G_{x,y}$ is the grey level at point (x,y) along the line profile and G_{blue} is the grey level of the blue region. The $G_{inc.}$ value for each point was then multiplied by $2\pi r$, where r is the distance from the center of the well or halo to the point (Fig. 1c). The overall discoloration (D) was calculated by adding the $2\pi r G_{inc.}$ values for each pink region from point (x,y_1) to point (x,y_2). Point (x,y_1) was at the boundary of the pink and blue regions, and point (x,y_2) was at the edge of the well (Fig. 1c). For each line profile, two D values were calculated, one for the pink region on either side of the well, which resulted in a total of four D values for each halo. All discoloration measurements were expressed in arbitrary units.

Identification of fungi. Three fungal isolates (A1.8, B2.14b, and C2.47) were identified by PCR amplification of the internally transcribed spacer (ITS) regions and the 5.8S ribosomal DNA (rDNA) or of the 18S rDNA. Primers ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGAT ATGC 3′) were used for amplification of the ITS regions and the 5.8S rDNA, and primers NS3 (5′ GCAAGTCTGGTGCCAGCAGCC 3′) and NS4 (5′ CTT CCGTCAATTCCTTTAAG 3′) were used to amplify part of the 18S rDNA subunit. The protocol used was the protocol described for amplification of the ITS regions by Webb et al. (59). Genomic DNA was extracted from freshly grown mycelia, frozen in liquid N_2, and crushed into a fine powder in a mortar. DNA was extracted by the method of Anderson et al. (1). Forward and reverse sequences and consensus sequences were compared to sequences in the EMBL fungal sequence database by using FASTA3 sequence homology searches. Morphological descriptions of identified isolates from previously published descriptions were used to confirm the identities.

RESULTS

Assay development. Two sets of petri dishes were used; one set contained CAS-UO_2^{2+} · HDTMA agar, and the other contained CAS-Fe^{3+} · HDTMA agar. An example of an assay plate is shown in Fig. 1a. To test the relationship between ligand concentration and discoloration, different amounts of DFO were added to the wells cut in the agar plates, and the discoloration was measured. Figure 2 shows the variation in discoloration with the amount of DFO for CAS-Fe^{3+} · HDTMA agar and CAS-UO_2^{2+} · HDTMA agar. All discoloration measurements were expressed in arbitrary units. A linear relationship between the discoloration of the dyed agars and the amount of siderophore added was observed for both the CAS-Fe^{3+} · HDTMA agar and the CAS-UO_2^{2+} · HDTMA agar ($R^2 = 0.992$ for the CAS-Fe^{3+} · HDTMA system; $R^2 = 0.9471$ for the CAS-UO_2^{2+} · HDTMA system). The ratios of discoloration in the CAS-UO_2^{2+} · HDTMA agar to discoloration in the CAS-Fe^{3+} · HDTMA agar with different amounts of DFO were calculated. There were no significant differences between the discoloration ratios with different amounts of DFO ($P > 0.05$, as determined by a t test).

To test the effect of acid on the dye, different amounts of DFO, citric acid, and HCl were added to the wells. Figure 3 shows the discoloration values for the different amounts of DFO, citric acid, and HCl. No discoloration was observed with HCl at any concentration (1, 0.1, or 0.01 M) on either the CAS-Fe^{3+} · HDTMA agar or the CAS-UO_2^{2+} · HDTMA agar. With DFO, discoloration was observed on both the CAS-Fe^{3+} · HDTMA agar and the CAS-UO_2^{2+} · HDTMA agar at all three concentrations (1, 0.5, and 0.1 mM). Discoloration was observed on the CAS-UO_2^{2+} · HDTMA agar with 0.1 and 0.01 M citric acid and on the CAS-Fe^{3+} · HDTMA agar with 0.1 M citric acid.

Application of the assay: siderophore production by soil isolates. (i) Isolation of soil microbes. Soil samples were obtained from three sites in Peak District National Park, and the soil pH was measured. The soil from site A (United Kingdom national grid reference SK 147 826) was a limestone grassland soil with a pH of 7.2 and an iron concentration of 3.32% (wt/wt). Site B (United Kingdom national grid reference SK 199 865) was a gritstone region, but the soil was well drained

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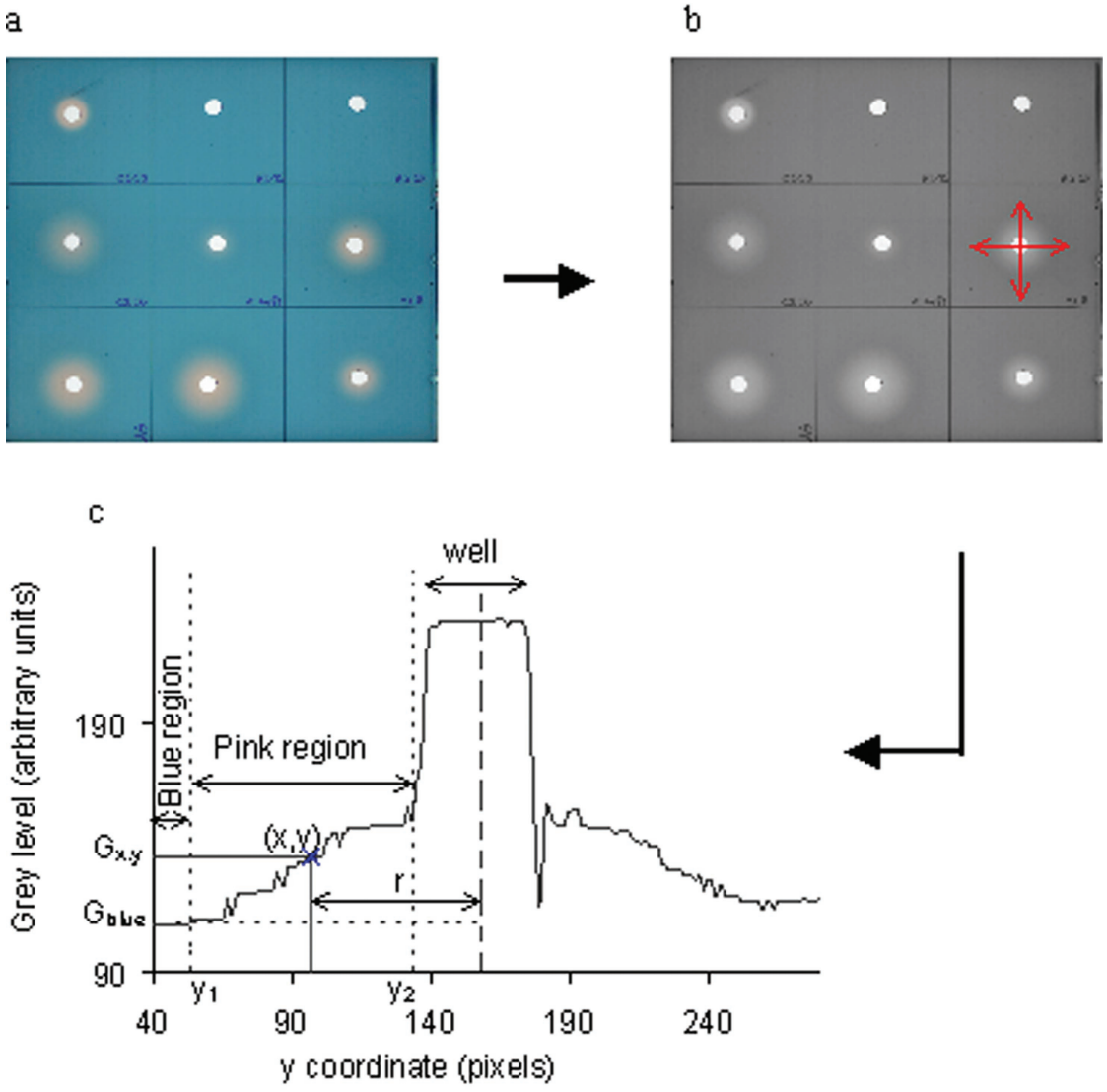

FIG. 1. Image analysis of the plates used in the assay for comparison of UO_2^{2+} complexation and Fe^{3+} complexation. (a) Assay plate; (b) greyscale image of an assay plate showing the line profiles examined (arrows); (c) typical line profile. (*x*,*y*) is a general point in the pink region.

because of the steep incline. The surface vegetation was *Pteridium aquilinum*, the soil pH was 4.1, and the iron concentration was 6.35% (wt/wt). Site C (United Kingdom national grid reference SK 088 930) was also a gritstone region, but the soil was poorly drained because of the flat topography. The surface vegetation was *Calluna vulgaris*, the pH of the soil was 4.1, and the iron concentration was 0.52% (wt/wt). A total of 113 morphologically different isolates were obtained from the soil samples; 63 isolates were obtained from site A, 30 isolates were obtained from site B, and 20 isolates were obtained from site C.

(ii) *A. flavescens* JG-9 assay. Figure 4 shows the results obtained with a typical assay plate. Samples of the iron-limited culture supernatants were added to the wells cut in the medium. If hydroxamate siderophores were present in a supernatant, then a halo of *A. flavescens* JG-9 appeared around the well as the siderophores diffused out into the surrounding

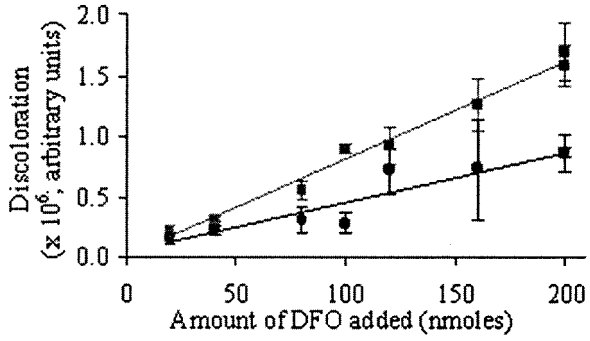

FIG. 2. Variation in the discoloration of CAS-Fe^{3+} · HDTMA agar and CAS-UO_2^{2+} · HDTMA agar with the amount of DFO added. The error bars indicate standard deviations ($n = 4$). Symbols: ▨, CAS-Fe^{3+} · HDTMA agar; ● CAS-UO_2^{2+} · HDTMA agar. Trend lines for each agar are shown.

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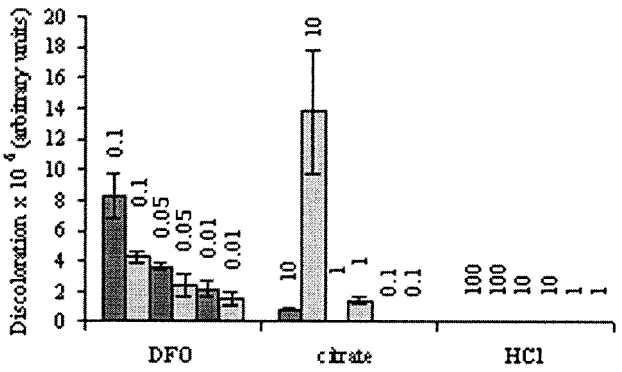

FIG. 3. Discoloration measured with different amounts of DFO, citrate, and HCl. The error bars indicate standard deviations ($n = 4$). The numbers above the columns are the amounts (in micromoles) of DFO, citrate, and HCl. Dark grey bars, CAS-Fe^{3+} · HDTMA agar; light grey bars, CAS-UO_2^{2+} · HDTMA agar.

medium. An isolate was defined as positive if a halo appeared on the assay plate. Site A (pH 7.2) had the lowest percentage of isolates that were positive as determined by the *A. flavescens* JG-9 assay (49%). For site B (pH 4.1), 67% of the isolates produced a positive response. Site C (pH 4.1) had the highest percentage of isolates that were positive (85%). There was not significant heterogeneity among the results for each site (as determined by a χ^2 test). Altogether, 60% of the isolates gave a positive response with the *A. flavescens* JG-9 assay.

(iii) Assay for comparison of UO_2^{2+} and Fe^{3+} complexation. Samples of the iron-limited culture supernatants were added to wells cut in the dyed agars. The discoloration (change in color from blue to pink) in the dyed agars caused by the supernatants was quantified. For each isolate that caused discoloration of the dyed agars, the discoloration ratio was calculated. A summary of the results is shown in Table 1. All of the supernatant samples that discolored the CAS-Fe^{3+} · HDTMA agar also discolored the CAS-UO_2^{2+} · HDTMA agar and vice versa. An isolate was considered positive if the supernatant discolored the dyed agars. Site B had the lowest percentage of isolates that were positive as determined by the CAS-based assay (67%). For site C, 75% of isolates produced a positive response. Site A had the highest percentage of isolates that were positive (79%). There was not significant heterogeneity among the results for each site (as determined by a χ^2 test). Altogether, 75% of the isolates tested gave a positive response with this assay.

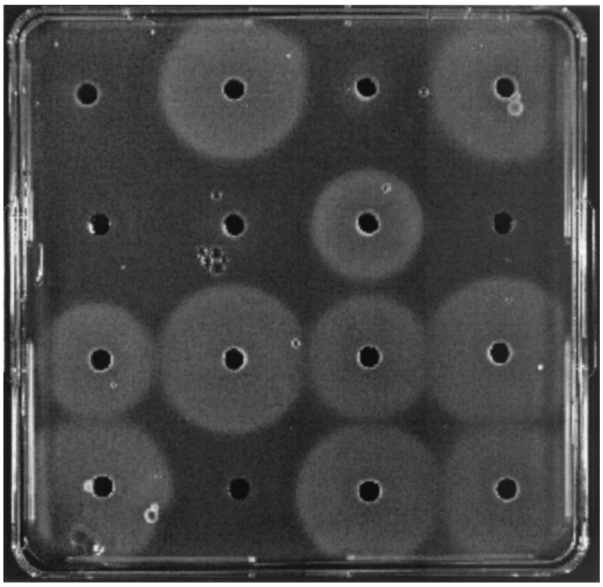

FIG. 4. Typical *A. flavescens* JG-9 assay plate.

TABLE 1. Results of the assay for the comparison of UO_2^{2+} complexation and Fe^{3+} complexation

Site	No. of isolates	No. of isolates positive[a]	No. of isolates with a discoloration ratio of ≥1
A	63	50	9
B	30	20	7
C	20	15	5
Total	113	85	21

[a] An isolate was considered positive if the supernatant discolored the dyed agar.

Table 1 also shows the number of isolates tested that produced a discoloration ratio of ≥1 (i.e., the number of isolates that caused the same or greater discoloration on the CAS-UO_2^{2+} · HDTMA agar as on the CAS-Fe^{3+} · HDTMA agar). For site A, 9 of the 50 isolates (18%) that gave a positive response had a discoloration ratio of ≥1. For site B, 7 of 20 positive isolates (35%) had discoloration ratios of ≥1, and for site C, 5 of 15 positive isolates (33%) had discoloration ratios of ≥1. There was not significant heterogeneity among the results for each site (as determined by a χ^2 test). Altogether, 21 of the 85 isolates that were positive (25%) had discoloration ratios of ≥1. Three isolates (A1.8, B2.14b, and C2.47) were selected for further study. The discoloration measured on each dyed agar and the discoloration ratio for each of these three isolates are shown in Table 2. All three isolates gave a positive response to the *A. flavescens* JG-9 assay.

(iv) Comparison of assay results. A total of 27 of the isolates (24 isolates from site A and 3 isolates from site B) were positive as determined by the CAS-based assay but negative as determined by the *A. flavescens* JG-9 assay. Ten isolates (five isolates from site A, three isolates from site B, and two isolates from site C) were positive as determined by the *A. flavescens* JG-9 assay but negative as determined by the CAS-based assay.

(v) Identification of fungi. Three isolates (isolates A1.8, B2.14b, and C2.47) were identified to the genus level by sequencing the ITS regions and 5.8S rDNA and the 18S rDNA

TABLE 2. Amount of discoloration and discoloration ratio for isolates A1.8, B2.14b, and C2.47 selected for further study

Isolate	Discoloration		Discoloration ratio
	CAS-Fe^{3+} · HDTMA	CAS-UO_2^{2+} · HDTMA	
A1.8	210,956 ± 30,987	275,472 ± 61,741	1.31 ± 0.35
B2.14b	93,720 ± 5,316	101,951 ± 17,449	1.09 ± 0.20
C2.47	143,684 ± 344,922	180,163 ± 16,249	1.25 ± 0.32

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and by examining the morphology. The sequences were compared to sequences in the EMBL fungal database. For B2.14b and C2.47 the matches with the highest levels of homology were with *Penicillium* and *Eupenicillium* species. This identification was confirmed by observation under a microscope of phialides arranged in a brushlike pattern with chains of spores extending from the phialides. This arrangement is typical of and found only in *Penicillium* and *Eupenicillium* species. For the isolate A1.8 18S rDNA, there were three matches with levels of homology greater than 90%: *Mucor mucedo*, *Mucor racemosus*, and *Pilobolus longipes*. For the ITS regions and 5.8S rDNA there were no matches with levels of homology greater than 81%, and thus the data were insufficient for accurate identification of the organism. A number of taxonomic keys were used (10, 19–21, 68), and a number of morphological characteristics were examined, including spore-forming structures, spores, growth rates, and color, which confirmed identification of this isolate as a *Mucor* species.

DISCUSSION

Assay development. The aim of this study was to develop a quick and simple assay to determine the relative effectiveness of different microbial ligands as chelators for UO_2^{2+} for use in screening large numbers of organisms. The widely used CAS assay for siderophores (44) is very simple, sensitive, and convenient. An additional advantage of this system is that it could easily be adapted to detect actinide complexation, as CAS has been used previously as a colorimetric reagent for uranium, for which it acts as a ligand (5, 46).

The CAS-Fe^{3+} · HDTMA dye can be used in two ways. A sample of a ligand can be added to the dye, and then the absorbance is measured. When a ligand is added, the absorbance of the dye at 630 nm decreases as Fe^{3+} is sequestered from the CAS complex. There is a linear relationship between the amount of ligand added to the dye and the absorbance of the dye. However, for CAS-UO_2^{2+} · HDTMA, there is no simple, reproducible relationship between absorbance and the amount of ligand added (41), probably because of the formation of colloids (5). Therefore, a spectrophotometric method with this CAS-based system could not be used to measure actinide complexation by siderophores.

CAS-Fe^{3+} · HDTMA can also be added to solid, iron-limited media. Organisms are grown on these media, and siderophore production is detected by the appearance of orange-pink haloes around colonies. When CAS-UO_2^{2+} · HDTMA was used instead, distinct haloes also appeared when UO_2^{2+} was removed from the dye complex by the secreted ligands (41).

An assay for actinide complexation was developed based on this orange-pink discoloration caused by secreted ligands. In this assay the discoloration of CAS-Fe^{3+} · HDTMA- or CAS-UO_2^{2+} · HDTMA-dyed agar caused by ligands was quantified. The effects of ligands on the two dyed agars were compared by calculating the ratio of the discoloration of the CAS-UO_2^{2+} · HDTMA agar to the discoloration of the CAS- Fe^{3+} · HDTMA agar; the higher the ratio, the more effective the ligand at chelating UO_2^{2+} relative to Fe^{3+}. The results showed that there was a regular reproducible relationship between the discoloration observed and the amount of ligand (in this case DFO) added and that the ratio of discoloration depended only on the ability of the ligand to chelate UO_2^{2+} and Fe^{3+} and was independent of the amount of ligand present. Therefore, this assay could be used to compare the abilities of different ligands to complex UO_2^{2+}.

The effect of acid on the dyed agars was also investigated. This experiment was performed to make sure that the agars were buffered sufficiently to prevent any changes in pH. Any reduction in pH would cause discoloration in the dyed agars and thus give false results. HCl did not cause any discoloration in either of the dyed agars, even at high concentrations (1 M). This shows that the buffer present was strong enough to prevent any change in pH which would cause discoloration. Discoloration was observed in both dyed agars with citric acid and DFO; both ligands can complex UO_2^{2+} and Fe^{3+} (2, 36, 45). These results prove that discoloration in the dyed agars is caused only by ligands that sequester the metal ion from the dye. However, these results also show that the assay detects any ligand capable of complexing Fe^{3+} or UO_2^{2+}, not just siderophores. Therefore, an additional assay was needed to detect the presence of hydroxamate siderophores. In this assay we used the hydroxamate siderophore auxotroph *A. flavescens* JG-9.

Application of the assay: siderophore production by soil isolates. The sites used were chosen because it was expected that iron would be unavailable in the soils, encouraging production of siderophores by the microorganisms present. The soil from site A was slightly alkaline, and the Fe^{3+} in the soil was expected to be present almost exclusively in highly insoluble forms and so be unavailable for uptake by microorganisms. The soils from sites B and C were more acidic but were rich in organic matter which could complex Fe^{3+}, reducing its availability to microorganisms (11, 18).

The isolates were grown in iron-limited liquid cultures, and two tests were carried out with the culture supernatants. In the first test, the presence of hydroxamate siderophores in each culture supernatant was detected by using *A. flavescens* JG-9. The second test was the assay in which UO_2^{2+} complexation and Fe^{3+} complexation were compared, which was used to compare the relative abilities of the ligands present in the supernatants to complex UO_2^{2+}. When the results of the two assays were compared, there were some isolates that were positive as determined by the CAS-based assay but negative as determined by the *A. flavescens* JG-9 assay. This shows that in these cases, there were ligands in the supernatant samples that could sequester Fe^{3+} and UO_2^{2+} from CAS but had no growth-promoting activity for *A. flavescens* JG-9. Most fungi produce more than one siderophore, and *A. flavescens* JG-9 responds to the great majority of fungal hydroxamate siderophores. Therefore, it is unlikely that all of the siderophores produced by an isolate would not be growth factors for *A. flavescens* JG-9.

It is probable that the organisms secreted organic acids or other, nonhydroxamate ligands which are capable of chelating Fe^{3+} and UO_2^{2+}. These acids would have a dual effect on Fe^{3+} availability in the soil by locally reducing the pH of the soil, thus increasing the solubility of Fe^{3+}, and by acting as chelating agents for Fe^{3+} (13). Fungi produce a wide variety of organic acids (14, 57, 58, 61) and have been found to secrete organic acids in response to low iron concentrations (6, 62).

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Ten isolates (five isolates from site A, three isolates from site B, and two isolates from site C) were positive as determined by the *A. flavescens* JG-9 assay but negative as determined by the CAS-based assay. It is possible that the organisms secreted compounds that have growth factor activity for *A. flavescens* JG-9 but cannot sequester Fe^{3+} and UO_2^{2+} from CAS, but it is unlikely. *A. flavescens* JG-9 is auxotrophic for hydroxamate siderophores, and so isolates that were positive as determined by the *A. flavescens* JG-9 assay must have produced hydroxamate siderophores. Such siderophores are very effective chelating agents for Fe^{3+}, forming highly stable complexes with formation constants in the range of 10^{30} (34), and they are also known to complex actinides effectively (2, 43). Any hydroxamate siderophores produced would be capable of removing Fe^{3+} from the CAS-Fe^{3+} · HDTMA complex. The second, more likely explanation is that the difference in the responses in the two assays reflects the relative sensitivities of the assays. *A. flavescens* JG-9 can be very sensitive to the presence of hydroxamate siderophores; it requires just ~0.5 ng of DFO ml^{-1} for half-maximal growth (29). With the CAS-based assay, the lowest amount of discoloration measured on the CAS-Fe^{3+} · HDTMA agar was equivalent to the discoloration observed with ~3.7 nmol of DFO in 200 μl or ~12 μg of DFO ml^{-1}, a concentration 4 orders of magnitude higher than the concentration of DFO required for half-maximal growth of *A. flavescens* JG-9. This suggests that the *A. flavescens* JG-9 assay is far more sensitive to the presence of hydroxamates than the assay in which UO_2^{2+} complexation and Fe^{3+} complexation are compared.

The results of the screening analysis were used to select three isolates for further study; one isolate was selected from each site. All three isolates produced hydroxamate siderophores as determined by the *A. Flavescens* JG-9 assay. In making the selection, we considered two factors. The first was the discoloration ratio. The aim was to find isolates that produced good chelators for UO_2^{2+}, so only those isolates with discoloration ratios of >1 were considered. The second factor was the amount of discoloration observed on the dye plates. Only isolates that produced a discoloration of > 90,000 on the CAS-Fe^{3+} · HDTMA plates (equivalent to the discoloration observed with ~50 μM DFO) were considered. This was to ensure that the organisms selected produced a reasonable amount of siderophore for further study. The isolates from sites B and C were identified as *Penicillium* species. These identifications were not surprising. *Penicillium* species are among the predominant fungi in the rhizosphere (7) and are known to produce a range of hydroxamate siderophores (3, 4, 26–28, 33, 63, 66, 67). The isolate from site A was identified as a *Mucor* species. Although mucoraceous fungi are commonly found in soil, they are not thought to produce hydroxamate siderophores (54, 65); rather, the polycarboxylate siderophore rhizoferrin is the common siderophore produced by the zygomycetes (53). However, Holzberg and Artis (23) detected hydroxamates in iron-limited cultures of the zygomycetes *Absidia corymbifera*, *Rhizopus arrhizus*, and *Rhizopus oryzae*, and Patil et al. (37) reported that *Cunninghamella blakesleeana* NCIM 687 produced ferrichrysin. There is also evidence which suggests that *Glomus* species may also produce hydroxamate siderophores (17).

In conclusion, our results show that the methods described here can be effectively used to screen a large number of fungi rapidly for the production of UO_2^{2+}-chelating ligands and that selectivity for UO_2^{2+} relative to selectivity for Fe^{3+} can be quantified by using the CAS-based assays, so that target species for detailed characterization of metal complexants can be identified. It is possible for mucoraceous fungi to produce hydroxamate siderophores.

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